Effect of EGF-Induced HDAC6 Activation on Corneal Epithelial Wound Healing

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Purpose. Epidermal growth factor (EGF) stimulates migration in corneal epithelial wound healing. The purpose of this study was to investigate the effect of EGF-induced α-tubulin deacetylation through activating HDAC6 on migration in corneal epithelial wound healing.

Methods. Human corneal epithelial (HCE) cells were cultured in DMEM/F12 medium containing 10% FBS in a 37°C incubator supplied with 5% CO_2. Western blot analysis was used to determine protein expression. Activity of HDAC6 was suppressed by trichostatin A (TSA) and by siRNA specific to HDAC6. Corneal epithelial cell migration was measured by using scratch-induced directional migration assay in cultured cells and by corneal epithelial debridement using a mouse whole-eye organ culture model.

Results. The authors found EGF stimulated corneal epithelial cell migration in wound healing by enhancing HDAC6 activity, resulting in the deacetylation of α-tubulin. EGF stimulated HDAC6 enzymatic activity and protein translocation toward the leading edge of the cell. Inhibition of HDAC6 activity by TSA significantly suppressed EGF-induced cell migration and delayed EGF-induced wound healing in epithelially debrided mouse corneas. In the meantime, knockdown of HDAC6 mRNA with specific siRNA effectively abolished EGF-induced deacetylation of α-tubulin, resulting in the inhibition of cell migration.

Conclusions. These results reveal an important mechanism that involves EGF-induced HDAC6 activation and α-tubulin deacetylation, subsequently affecting corneal epithelial migration in the wound-healing process. (*Invest Ophthalmol Vis Sci.* 2010; 51:2943–2948) DOI:10.1167/iovs.09-4639

The reversible acetylation targeting to both histone and nonhistone proteins plays important roles in posttranslational modifications of proteins. The balance between acetylation and de-acetylation is maintained by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Most HATs and HDACs are localized inside nuclei and regulate the acetylation of nuclear proteins.[1] In the nuclei, histone acetylation generally induces chromatin relaxation and gene transcription, whereas nonhistone acetylation functions widely, depending on its targeting proteins.[2,3] Interestingly, HDAC6 is one of the exceptional members in the HDAC family for its unique cytosol localization and enzymatic activity for deacetylation of several important cytosol proteins, including α-tubulin, Hsp90, and cortactin.[4–6] HDAC6 involves both microtubule- and microfilament-dependent cell motilities by regulating the acetylation level of these targeted proteins. Most evidence of the defective cell mobility found in HDAC6 knockout mice indicates that HDAC6 activity is essential for cell motility.[7,8]

Cell migration is involved in significant physiological and pathologic activities, such as wound healing and cancer metastasis. It has been shown that HDAC6 activity is associated with cancer invasion,[1,9,10] thus representing an attractive target for cancer chemotherapy.[11] However, accelerated cell migration is favored for wound healing and tissue repair. For example, corneal wound healing requires proper activities of cell migration that are essential for successful reepithelialization.[12] In the corneal epithelial wound-healing process, epithelial migration is initiated by microtubule and microfilament reorganization and is continued by microtubules forming distinct weblike patterns while circumferential microfilament bundles form lamellipodia, filopodia, and prominent stress.[12–14]

A recent study shows that HDAC inhibitors block the migration of corneal stromal cells; however, there are no reports indicating the role of HDAC6 in regulating cell migration through altering the acetylation of cytoskeleton proteins in corneal epithelial wound healing.[15] Based on the evidence that acetylation of cytoskeleton-related proteins, such as α-tubulin and cortactin, is closely associated with cell migration, we hypothesized that corneal epithelial wound healing involves the regulation of acetylation modifications. In the present study, we were interested in determining the role of HDAC6 activation and acetylation modification of cytoskeleton proteins in corneal epithelial wound healing. It is likely that HDAC6 is involved in growth factor-induced cell migration because HDAC6 overexpression could promote serum-induced cell migration,[6] and PDGF-induced Rac-1 activation is absent in HDAC6 knockout mice.[8] We found that EGF stimulation significantly enhanced the deacetylase activity of HDAC6, presenting a novel mechanism and detailing how growth factors regulate HDAC6 function. In addition, we demonstrated significant evidence for the first time to support the notion that corneal epithelial wound healing was dampened by the inhibitor of deacetylases and by knocking-down HDAC6. We also found that there was an enhanced activity of HDAC6 and a reduced level of acetylated α-tubulin in response to EGF stimulation in corneal epithelial cells. Thus, our study results contribute to a theory responsible for initiating cell migration in corneal wound healing.

Materials and Methods

Cell Culture and Reagents

Human corneal epithelial cells, including Araki-Sasaki SV-40 transformed HCE and telomerase-immortalized HTCE cells,[16] were grown in DMEM/F-12 (1:1) culture medium containing 10% FBS and serum-free medium supplied with growth factors in conditions of 5% CO_2 at 37°C, respectively (Defined keratinocyte-SFM; Invitrogen, Carlsbad, CA). Cells were rinsed three times with PBS and then cultured in their respective media without serum or supplements for 24 hours for

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synchronization. Cells were subcultured by digestion of 0.05% trypsin-EDTA followed by soybean inhibitor treatment. Culture media were replaced every 2 days. EGF and TSA were purchased from Sigma (St. Louis, MO). Nonspecific siRNA and HDAC6 siRNA (sc-35544) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A transfection kit was used to transfect the cells (HiPerFect; catalog no. 301705; Qiagen, Valencia, CA). Primary antibodies were purchased as follows: mouse anti-acetylated-tubulin (T6793) from Sigma and rabbit anti-tubulin and anti-HDAC6 (SC5546 and SC11420) from Santa Cruz Biotechnology.

HDAC6 Activity Assay

HDAC6 protein was immunoprecipitated from HCE cells using specific antibody against HDAC6 (Santa Cruz Biotechnology) and was incubated with HDAC colorimetric substrate that comprises an acetylated lysine side chain to determine HDAC6 activity. For immunoprecipitation, cells (5×10^7) were incubated in 1 mL lysis buffer (20 mM Tris pH 7.5, 137 mM NaCl, 1.5 mM, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, and 10 μg/mL aprotinin) on ice for 10 minutes. Cell lysates were sonicated and centrifuged at 13,000*g* for 10 minutes at 4°C. Supernatants were incubated with antibodies against HDAC6 at 4°C overnight. Immunocomplexes were recovered by incubation with 40 μL 25% protein A/G-Sepharose (Santa Cruz Biotechnology). Immunocomplex beads were washed twice with lysis buffer, dissolved in 1% SDS lysis buffer, and subjected to HDAC6 enzymatic activity assay using a kit purchased from (BioVision, Mountain View, CA). According to the manual, HDAC6 protein precipitated from HCE cells was incubated with HDAC colorimetric substrate at 37°C for 3 hours to deacetylate and sensitize the substrates. In the second step, the assay was stopped by the addition of 10 μL lysine developer and was incubated at 37°C for 30 minutes to produces a chromophore. Samples were read using an ELISA plate reader (LabSystem Multiskan MCC/340; Fisher Scientific, Pittsburgh, PA) at a wavelength of 405 nm.

Immunostaining Experiments

HCE cells were grown on glass slides and treated as indicated in the figure legends. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% to 0.3% Triton X-100, and blocked with 5% BSA before incubation with the mouse monoclonal antibody against acetyl-α-tubulin (Sigma) at 4°C overnight. Slides were washed with PBS and incubated with FITC-conjugated goat anti-mouse IgG antibody (Invitrogen). Cells were then examined under a fluorescence microscope.

Western Blot Analysis

Cells (2×10^5) were lysed in sample buffer containing 62.5 mM Tris-HCl pH 6.8, 2% wt/vol sodium dodecyl sulfoxide, 10% glycerol, 50 mM dithiothreitol, and 0.01% wt/vol bromophenol blue. Proteins were electrotransferred to polyvinylidene difluoride membranes blocked in 5% nonfat milk in TBS-0.1% Tween 20 (TBS-T) for 1 hour at room temperature (RT) and then incubated with the primary antibodies at 4°C overnight. Horseradish peroxidase–conjugated secondary antibody was applied in TBS-T buffer for 1 hour at RT. Western blot analysis was developed with the use of enhance chemiluminescence (ECL Plus; Santa Cruz Biotechnology) and visualized by exposure of X-ray films.

Corneal Wound Healing and Migration Assays

Scratch-Induced Directional Migration Assay. The cell migration rate was determined in HCE and HTCE in culture. A white pipette tip was used to make a cross-stripe scrapes. Wound closure was measured using an inverted microscope after a time course. The migration rate was represented by the difference between the width of the scratching area before and after culture.

Whole-eye Organ Culture Model. Corneal epithelial wound healing was performed by removing the corneal epithelial layer using a corneal rust ring remover (Alger brush; Alger, Lago Vista, TX) with a 0.5-mm burr under a dissecting microscope without damaging the Bowman's membrane. It has known that reepithelialization of the cornea in wound healing involves coordinated cellular responses, including cell migration, proliferation, and differentiation. The experiment was designed to examine cell migration in wound healing in the mouse model. A smaller corneal epithelial wounding area (0.5-mm diameter) was performed to allow the epithelial layer to heal within a few hours; we predicted cell migration would play a predominant role within this relatively short period. The corneal epithelial wound healing rate was measured in the absence and presence of EGF or EGF plus TSA. The mouse eye was dissected and placed in culture wells, corneal side facing up, in medium containing 1% antibiotic/antimycotic solution at 37°C and 5% CO_2. The rate of epithelial healing in whole-eye organ culture was measured immediately after wounding. Lesions of the eyes were topically stained with fluorescein (fluorescein sodium 1.0% wt/vol) and photographed by a confocal microscope (Leica, Wetzlar, Germany). All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research using protocols approved and monitored by the Animal Care Committee of LA BioMed at UCLA School of Medicine.

Statistical Analysis

Western blot signals in the films were scanned digitally, and optical density was quantified with image calculator software (CS4; Adobe Photoshop, Mountain View, CA). Data were shown as original values for the scratch gap closure in the culture dishes in triplicate and were repeated at least three times or for measured wounding region in the cornea as mean ± SE. Significant differences between the control group and treated groups were determined by one-way ANOVA and Student's *t*-test ($P < 0.05$).

Results

EGF-Induced Increase in HDAC6 Activity

To investigate the effect of EGF on HDAC6 function, we first examined the distribution of HDAC6 in cultured cells. EGF appeared to induce membrane ruffling at cell edges, indicating increased migratory ability in EGF-stimulated HTCE cells, with ruffles positively immunostained for HDAC6 protein with a specific antibody (Fig. 1A). In contrast, this activity and HDAC6 staining were barely visible in control cells, possibly because of low cell migration. Western blot analysis revealed that the HDAC6 protein level was not significantly changed in cells induced with various dosages of EGF (Fig. 1B). Next, HDAC6 activity was measured by using immunocomplex enzymatic assays, and the results were compared between cells with and without EGF stimulation. Total amounts of immunoprecipitated HDAC6 protein (IP-HDAC6) were presented as a loading control. In EGF-induced cells, EGF stimulation significantly enhanced HDAC6 activity after a time course (Fig. 1C). TSA, a pan-inhibitor of various HDACs, was applied to cell culture before EGF stimulation. Results showed that the EGF effect on HADC6 activation was significantly blocked by TSA (Fig. 1D). They also indicated that HDAC6 is associated with membrane fraction on EGF treatment and that EGF-induced acceleration of cell migration was dependent on its ability to activate HDAC6 in human corneal epithelial cells.

Effect of Inhibiting HDAC on Cell Migration

The effect of inhibiting HDACs with TSA on cell migration was investigated by cell scratch assays. Treatment of HCE cells with EGF promoted cell migration and wound closure in both dose- and time-dependent manners (Fig. 2A). Application of TSA induced a delay of corneal epithelial cell wound closure in normal culture conditions, suggesting that

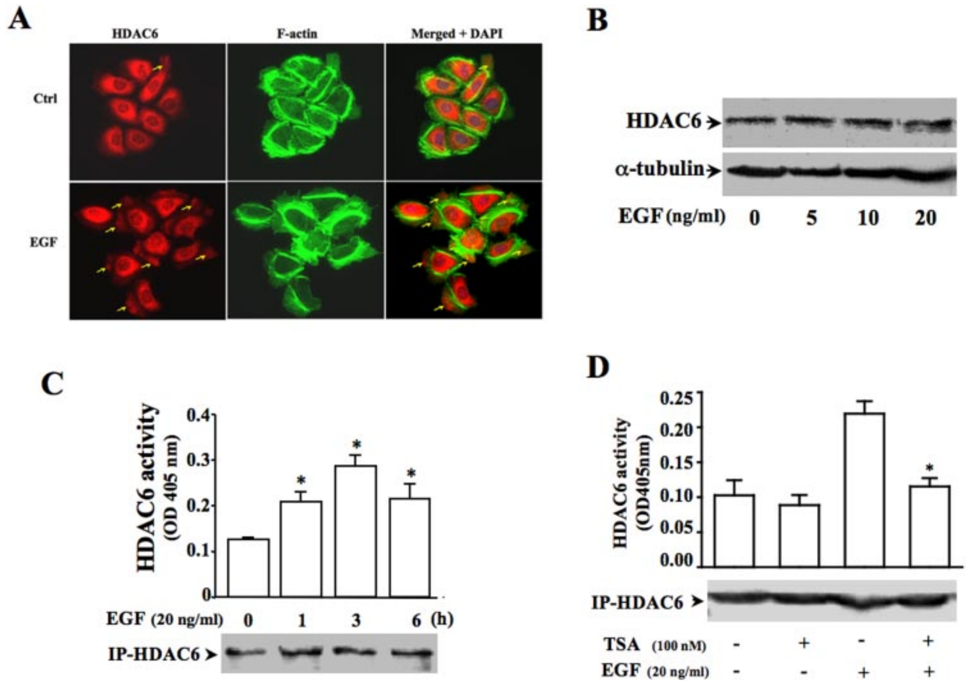

Figure 1. Effect of EGF on HDAC6 expression and activity. (**A**) EGF-induced HDAC6 translocating to the membrane of the cell edge in HTCE cells. *Arrows*: membrane translocations of HDAC6 proteins. (**B**) Effect of various EGF concentrations on expressions of total cellular HDAC6 and α-tubulin. (**C**) Effect of EGF stimulation on HDAC6 enzymatic activity. (**D**) Effect of TSA on EGF-induced HDAC6 activation. HCE cells in triplicate were exposed to different treatments, as indicated, for 5 hours. Western blot analysis was performed to detect protein expression. HDAC6 was immunoprecipitated (IP) from cells stimulated with and without 20 ng/mL EGF or TSA for 5 hours. HDAC6 activity was measured by using immunocomplex enzymatic assay kits in three independent experiments. *Statistical significance between HCE cells with and without treatments ($n = 3$; $P < 0.05$).

TSA suppressed cell migration (Fig. 2B). Interestingly, we found that EGF-promoted increases in cell migration were abrogated by the inhibition of HDACs with TSA (Fig. 2C). Therefore, these results indicated that a reduced level of protein acetylation was required for corneal epithelial cell migration in the wound-healing process in the absence and presence of EGF stimulation.

Involvement of HDAC6 in HCE Cell Migration

The inhibitory effect of TSA in HCE cells was not specific and prompted us to further investigate which of the HDAC subtypes is involved in corneal epithelial cell migration. HDAC6-specific siRNA was used in HCE cells to knock down HDAC6 mRNA. Western blot analysis revealed that HDAC6 expression was markedly decreased by siRNA transfection (Fig. 3A). Knockdown of HDAC6 evidently decreased HCE cell migration in the absence and presence of EGF stimulation, resembling the inhibitory effect of TSA on cell migration (Figs. 3B, 3C). In addition, nonspecific siRNA-transfected cells in parallel experiments were used as the controls for comparison with HDAC6-specific siRNA-transfected cells. Taken together, we concluded that HDAC6 was

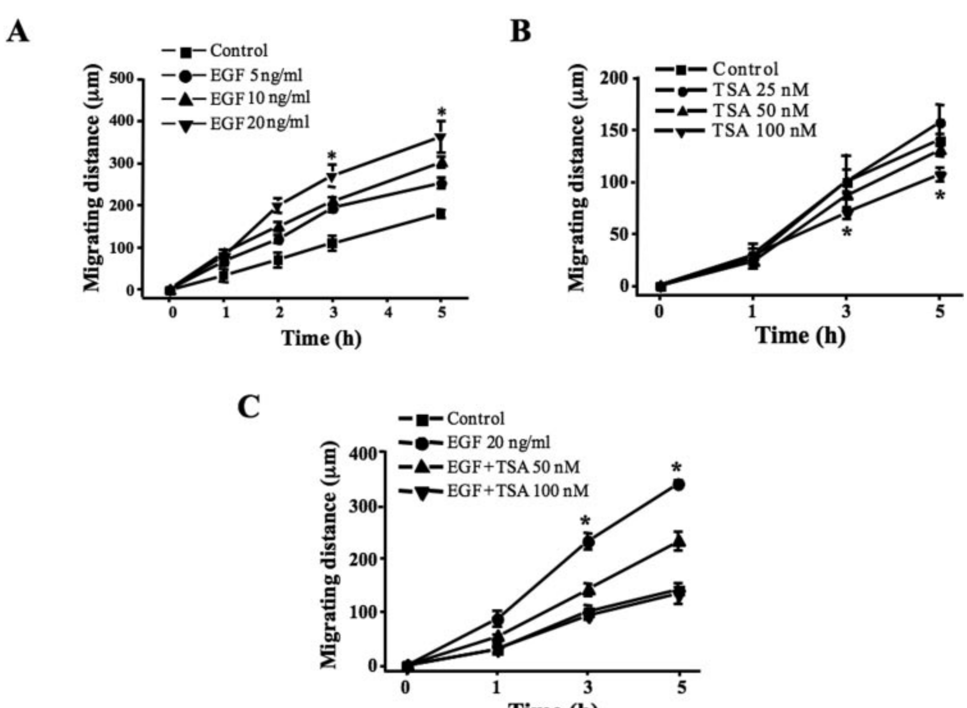

Figure 2. Effects of EGF and inhibiting HDAC6 on cell migration. (**A**) Dose-dependent response of EGF-induced acceleration of wound closure. (**B**) Effect of different concentrations of TSA on cell migration. (**C**) Inhibitory effect of TSA on EGF-stimulated wound closure acceleration. HCE cells were grown in normal culture conditions for 48 hours to reach 90% confluence. Wounds were made by a linear scrape 700-μm wide using a 200-μL pipette tip. The cells were washed with PBS to move debris and were re-fed with the fresh medium. The width of wounding areas was measured in triplet culture dishes and repeated three times at the indicated time. *Statistical significance between HCE cells with and without treatment ($n = 3$; $P < 0.05$).

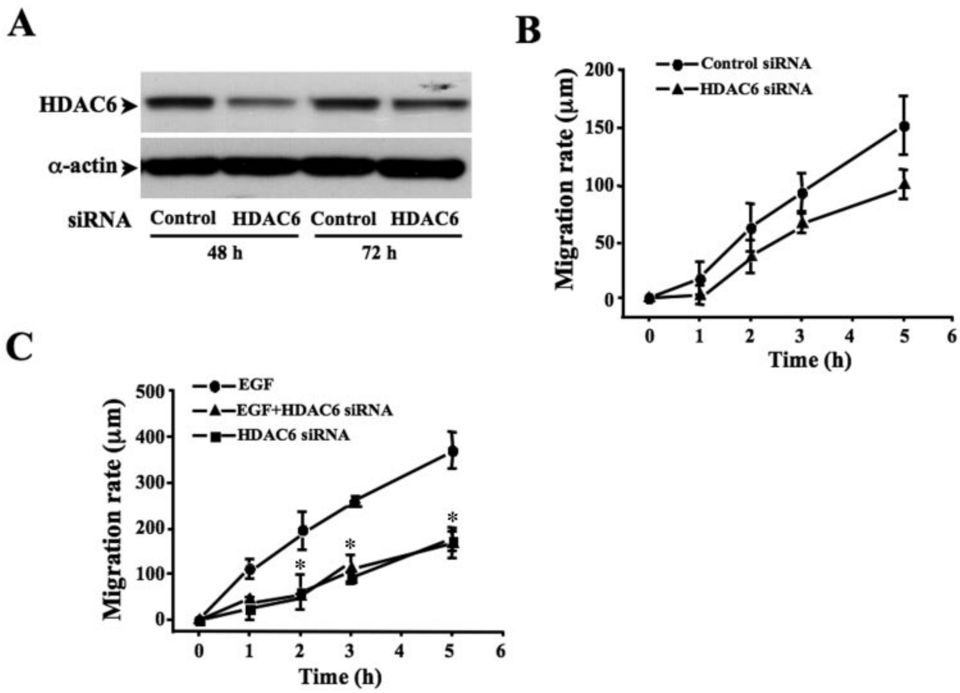

FIGURE 3. Effect of knocking down HDAC6 on cell migration. (**A**) Suppressed HDAC6 protein expression caused by knocking down HDAC6 mRNA in 48 and 72 hours. (**B**) Effect of knocking down HDAC6 on cell migration. (**C**) Effect of knocking down HDAC6 on EGF-stimulated cell migration. HCE cells were transfected in triplet culture dishes with either nonspecific siRNA for controls or HDAC6-specific siRNA, and cells were harvested and subjected to Western blot analysis 48 hours after transfection. *Statistical significance on comparison of cell migration rates between NS-transfected and HDAC6 siRNA-transfected cells from three independent experiments ($n = 3$; $P < 0.05$).

one of the HDAC subtypes required for cell motility in corneal epithelial wound healing.

Requirement of HDAC6 in Mediating Deacetylation of α-Tubulin

The role of HDAC6 in mediating deacetylation of cytoskeletal microtubules was determined in HCE cells by suppressing HDAC6 with TSA and by knocking down HDAC6 using specific siRNA. We found that EGF stimulation dramatically reduced the level of acetylated α-tubulin in HCE cells, whereas the total amount of α-tubulin protein expression was not affected (Fig. 4A). EGF-induced α-tubulin deacetylation was significantly suppressed by the inhibition of HDAC6 activity with TSA (Fig. 4B). In line with the results of Western blot analysis, immunostaining experiments revealed a decrease in acetylated α-tubulin in EGF-induced cells in 5 hours. In contrast, there was a tremendous increase in immunoreactive activities of acetylated α-tubulin in TSA-treated cells (Fig. 4C). In addition, the knockdown of HDAC6 with specific siRNA effectively abolished EGF-induced deacetylation of α-tubulin (Fig. 4D). Thus, the inhibition

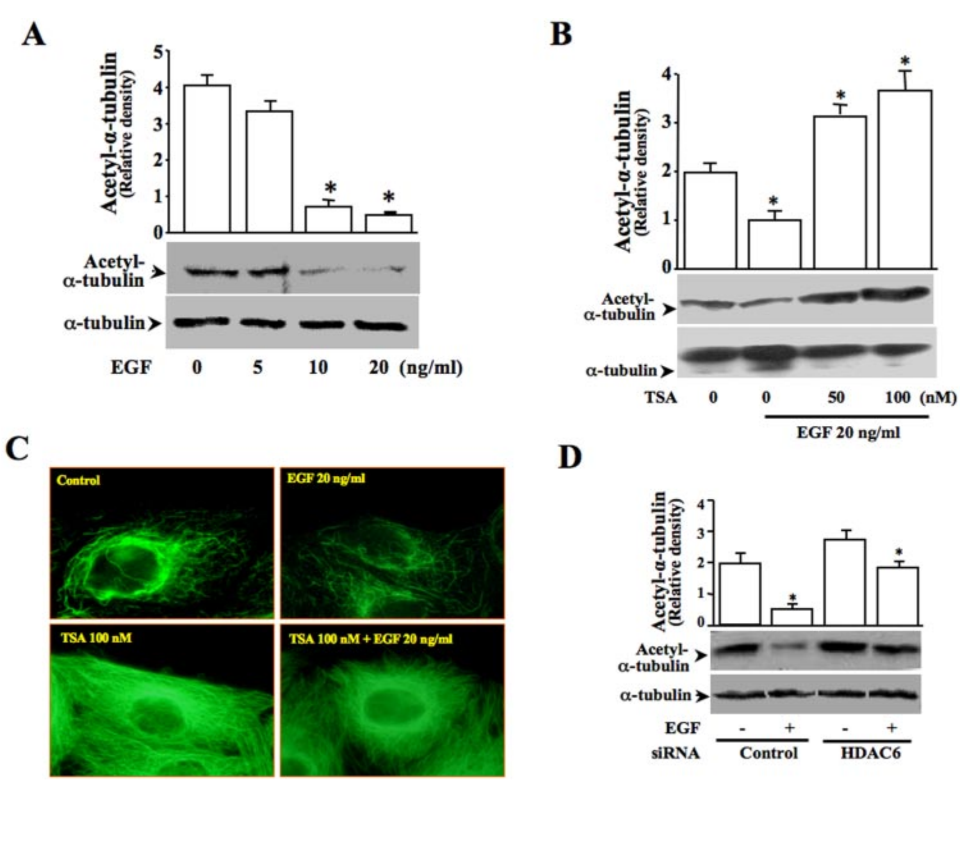

FIGURE 4. Effects of EGF and TSA treatments on acetylation of α-tubulin. (**A**) Dose-dependent effect of EGF-induced deacetylation of α-tubulin. (**B**) Dose-dependent inhibition of α-tubulin deacetylation by TSA. (**C**) Effects of EGF, TSA, and EGF plus TSA on cellular levels of acetylated α-tubulin detected by immunostaining. (**D**) Effect of knocking down HDAC6 mRNA in 48 hours on EGF-induced deacetylation of α-tubulin. HCE cells in triplet culture dishes were synchronized by serum starvation for 24 hours and were treated with and without EGF, TSA, and EGF plus TSA for 5 hours. Levels of acetylated α-tubulin were detected by Western blot analysis or by immunostaining using specific anti–acetylated α-tubulin antibodies. Total cellular α-tubulin amount was detected by anti–α-tubulin antibody in control experiments. Data were collected from three independent experiments. *Statistical significance ($n = 3$; $P < 0.05$).

of HDAC6 activity by either TSA- or HDAC6-specific siRNA not only increased the basal level of acetylated α-tubulin, it significantly suppressed EGF-induced decreases in the acetylation of α-tubulin, indicating that HDAC6 mediates EGF-induced deacetylation of α-tubulin to affect corneal epithelial migration in the wound-healing process.

Effect of Inhibiting HDAC on Wound Healing

The effect of EGF-induced HDAC6 activation on corneal epithelial wound healing was evaluated in the absence and presence of TSA in cultured mouse corneas. Debridement of corneal epithelial cells was made in a 0.5-mm diameter area near the center of the excised mouse cornea. The corneas were incubated for 5 hours in the absence (for controls) and presence of EGF, TSA, or EGF plus TSA (Fig. 5A). At the end of healing time points, the wounding area in each group was stained with 1% fluorescein and photographed with a Leica confocal microscope. As shown in Figure 5B, EGF stimulation accelerated the corneal epithelial wound-healing process, but the EGF effect on promoting wound healing was significantly blocked by 100 nM TSA. In addition, TSA, when it was applied alone, delayed the rate of corneal epithelial healing. These results indicated that an increased level of acetylation could result in the slowing of corneal epithelial wound healing.

Discussion

Human HDAC6 is stably anchored in cytoplasm, unlike nuclear subtypes in the HDAC family,[1,17] providing itself a unique function in mediating cell adhesion and migration in various cell types, such as fibroblasts, T lymphocytes, and cancer cells.[10,15,17–21] In the present study, we aimed to define the role of HDAC6 in corneal epithelial wound healing. Our results revealed a new mechanism indicating that EGF accelerated cell migration by inducing the deacetylation of cytoskeletal microtubules through the activation of HDAC6. We found that HDAC6 is required for corneal epithelial cell migration in the wound-healing process. HDAC6 inhibitor TSA was able to dampen cell migration, resulting in the slowing of corneal epithelial wound healing in mouse corneas. The effect of TSA is consistent with the results obtained from those transfected cells in which HDAC6 activity was knocked down by HDAC6-specific siRNA.

Cytoskeleton reorganization is vital for initiating cell migration in corneal epithelial wound healing.[12,13] During the process of cytoskeleton reorganization, posttranslational modification of cytoskeletal proteins might be a key factor for mediating the fast rearrangement of filament network.[22] In the corneal epithelial wound-healing process, protein phosphorylation that mediates the activation of several signaling pathways, such as the ROCKs-Rho–associated pathway and the ERK-FAK pathway, is the major modification affecting corneal epithelial cell migration.[23–25] We believe that EGF-induced signaling pathways are upstream events that control HDAC6 activity to mediate EGF-induced acceleration of corneal epithelial cell migration and wound healing. We found that the PD98059 inhibitor specific to the ERK-MAP kinase pathway—important for mediating the EGF effect—abrogated EGF-enhanced HDAC6 activity (data not shown). This is necessary to further determine the phosphorylation status of HDAC6 and to clarify the protein interactions that are responsible for regulating HDAC6 activity in EGF-stimulated cells in future studies.

HDAC6 targets several cytosol proteins to mediate cell motility. One of the important HDAC6-targeting proteins is α-tubulin, which is the first microtubule regulated by HDAC6-mediated deacetylation.[6] In addition, HDAC6 can influence microfilament organization by altering the acetylation status of cortactin and HSP90.[8] The hypoacetylation of cortactin promotes its translocation to the cell periphery, enhances its association with F-actin, and increases cell motility.[4] In response to PDGF stimulation, both HDAC6 and hypoacetylated-HSP90 induced by HDAC6 are recruited to the membrane and to collaboratively activate Rac1-GTP, leading to membrane ruffle formation and cell migrating.[8]

HDAC6-mediated cell motility has been investigated in cancer cells, especially breast cancer cells. Hsp90 is one of the important targets that mediate HDAC6-promoted tumor invasion.[9,20] In addition, HDAC6 is required for efficient oncogenic tumorigenesis[21] and for TGF-β1–induced epithelial-mesenchymal transition.[26] Cell proliferation and migration are the prosurvival cellular events favored in corneal epithelial wound healing, in contrast to cancer. Consistently, we have shown that the inhibition of HDAC6 activity by TSA, which has potent efficiency in suppressing cancer, results in a negative effect to improve corneal epithelial wound healing. Thus, a strategy to enhance the deacetylation enzymatic activity of HDAC6 to reduce the acetylation of cytoskeleton is favored in corneal epithelial wound healing. Topical application of growth factors, such as EGF, PDGF, and FGF, has been explored for the promotion of wound healing.[12] In the present study, we found that HDAC6 activity was increased in response to EGF stimulation and was accompanied by a reduction in acetylated α-tu-

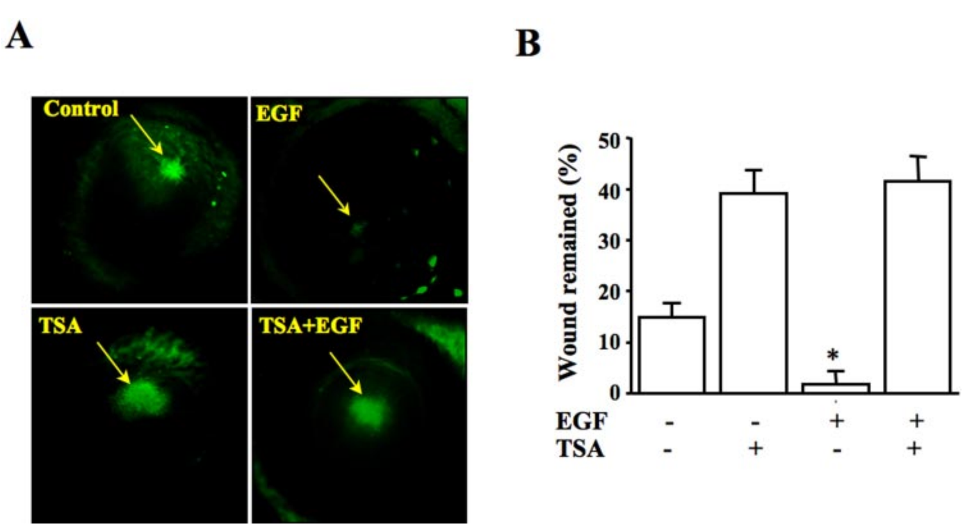

Figure 5. Effect of inhibiting HDAC6 activities with TSA on corneal epithelial wound healing. (**A**) Corneal epithelial wound healing determined in the absence and presence of EGF or TSA and EGF or EGF plus TSA. (**B**) Statistical analysis of corneal epithelial wound healing under different conditions. Corneal epithelial debridement was made in the defined area of the cornea by using a corneal lesion with Alger brush corneal rust ring remover and a 0.5-mm burr, followed by fluorescein staining (1.0% wt/vol). Delayed wound healing of mouse corneal epithelia was determined by comparing control, EGF-, and TSA-treated groups 5 hours after wounding. Experiments were conducted in the left eyes for comparison with control right eyes of the same mice (n = 4 mice per experiment; $P < 0.05$). *Significant differences.

bulin. In the present study, inhibiting HDAC6 activity by TSA and HDAC6-specific siRNA transfection suppressed EGF-enhanced cell migration, providing additional data regarding the therapeutic effect of EGF in corneal epithelia wound healing.

The involvement of HDC6 in EGF-induced acceleration of corneal epithelial cell migration prompted important questions, leading us to investigate the acetylation level of α-tubulin in EGF-stimulated HCE cells. We observed a reduction of acetylated α-tubulin through the activation of HDAC6 in EGF-induced cells, indicating the role of α-tubulin acetylation in mediating EGF-accelerated corneal epithelial wound healing. The effect of altered α-tubulin acetylation on cell motility might have resulted from acetylation-induced changes of microtubule stability; however, the underlying mechanism of the altered α-tubulin acetylation contribution to cell migration is still largely undefined.[6,27] Furthermore, HDAC6-mediated deacetylation of cortactin and Hsp90 affects microfilament function in cell motility.[8] The rearrangement of microfilament, including the formation of lamellipodia and filopodia, will be of future interest in the study of corneal epithelial wound healing.

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